NICEATM-ICCVAM# International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions
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The International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: introduction and summary

William S Stokesa*, Jodie Kulpa-Eddyb, Richard McFarlandc

aNational Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA, bUnited States Department of Agriculture-Animal and Plant Health Inspection Service-Veterinary Services, Riverdale, Maryland, USA, cUnited States Food and Drug Administration - Center for Biologics Evaluation and Research, Rockville, Maryland, USA

Abstract

Vaccines contribute to improved animal and human health and welfare by preventing diseases and deaths from infectious diseases. However, testing necessary to ensure vaccine effectiveness and safety can involve large numbers of animals and significant pain and distress. NICEATM and ICCVAM recently convened an international workshop to review the state of the science of available alternative methods and approaches that can further reduce, refine, and replace the use of animals for human and veterinary vaccine potency and safety testing, and to identify research, development, and validation efforts necessary to further advance new and improved alternative methods. Workshop participants identified human and veterinary vaccines that should have the highest priority for future efforts. Prioritization criteria included testing that involves significant pain and distress, large numbers of animals, and pathogens that are dangerous to people and animals. Participants noted that in vitro antigen quantification assays have replaced animals for potency testing for some killed vaccines, and recommended that this approach be expanded to other vaccines. Recommendations to support more humane animal use included development and use of humane endpoints for all challenge tests, development of serologic assays to replace challenge tests, and development of in vitro toxin neutralization tests (TNT) to replace in vivo TNTs. Workshop participants recommended several approaches that might further reduce the number of animals required for specific potency tests. Participants also recommended priority vaccines for which alternative safety testing methods should be pursued and that would have the greatest impact on avoiding pain and distress and reducing animal numbers. Finally, workshop participants recommended enhanced international harmonization and cooperation efforts and closer collaborations between human and veterinary researchers to expedite progress. Implementation of the workshop recommendations is expected to advance new methods for vaccine testing that will reduce animal use, benefit animal welfare, and ensure continued and improved protection of human and animal health.

* The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods and the Interagency Coordinating Committee on the Validation of Alternative Methods
* Corresponding author e-mail address: stokes@niehs.nih.gov
The views expressed in this manuscript are those of the authors and do not necessarily represent the official position of any Federal agency, the European Commission, or other organization.

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1. Background

Vaccines contribute to improved animal and human health and welfare by preventing illnesses and deaths in people and animals that can result from infectious diseases. However, testing necessary to ensure vaccine effectiveness and safety can involve large numbers of animals and significant animal pain and distress. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which promote alternative methods that can reduce, refine (less pain and distress) and replace animal use in testing, recently identified alternative test methods for vaccine potency and safety testing as one its four highest priorities [1]. NICEATM and ICCVAM subsequently convened an international workshop to review the state of the science of available alternative methods for human and veterinary vaccine potency and safety testing, and to identify research, development, and validation efforts necessary to advance new and improved methods that can further reduce, refine and replace animal use for vaccine testing.

ICCVAM is a permanent Federal interagency committee charged by law with promoting the scientific validation, regulatory acceptance, and national and international harmonization of test methods that accurately assess the safety of chemicals and products while reducing, refining (less pain and distress), and replacing animal use. The committee evaluates new, revised, and alternative methods with regulatory applicability, and forwards formal recommendations to Federal agencies for acceptance decisions. ICCVAM members include 15 U.S. Federal regulatory and research agencies that require, use, generate, or disseminate safety testing data, including the U.S. Department of Agriculture (USDA), which regulates veterinary vaccines, and the U.S. Food and Drug Administration (U.S. FDA), which regulates human vaccines. NICEATM, a Center in the National Institute of Environmental Health Sciences (NIEHS) at the National Institutes of Health, administers ICCVAM, provides scientific and operational support for ICCVAM-related activities, and conducts international validation studies on promising new safety testing methods. NICEATM and ICCVAM serve a critical role in translating research advances from the bench into standardized safety tools that can be used in regulatory practice to prevent disease and injury.

In order to promote and advance the development and use of scientifically valid alternative methods for human and veterinary vaccine testing, NICEATM and ICCVAM organized the International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions. The workshop, held on September 14-16, 2010 at the National Institutes of Health in Bethesda, Maryland, U.S.A., was organized in partnership with the European Centre for the Validation of Alternative Methods (ECVAM), the Japanese Center for the Validation of Alternative Methods (JaCVAM) and Health Canada, and was co-sponsored by the Society of Toxicology.

This workshop summary provides an overview of the workshop discussions and summarizes the workshop conclusions and recommendations. Additional details can be found in the workshop reports [2, 3, 4, 5, 6, 7] and individual speaker manuscripts that follow in these workshop proceedings.

2. Goals and organization of the workshop

The goals of the International Workshop were to: (1) identify and promote the implementation of currently available and accepted alternative methods that can reduce, refine and replace the use of animals in vaccine potency and safety testing; (2) review the state of the science of alternative methods and identify knowledge and data gaps that need to be addressed; and (3) identify and prioritize research, development and validation efforts necessary to address these gaps and to advance alternative methods that can ensure continued protection of human and animal health.
Expert scientists and regulatory authorities from the United States, Europe, Canada, and Japan were invited to present plenary lectures on key topics related to vaccine lot release potency and safety testing, which formed the basis for further discussions in workshop breakout sessions. Speakers reviewed the current state of the science and availability of alternative test methods that can reduce, refine (decrease or eliminate pain and distress), and replace the use of animals for human and veterinary vaccine post-licensing potency and safety testing. The workshop provided a unique opportunity for interested stakeholders from both the human and veterinary vaccine fields and from different geographic regions to exchange important insights on the current similarities and differences in vaccine potency and safety testing procedures and approaches. Nearly 200 scientists from 13 countries attended the workshop.

The workshop opened with a plenary session in which expert scientists and regulatory authorities outlined the importance of vaccines to human and animal health, and described national and international regulatory testing requirements and guidelines for human and veterinary vaccines. Daily plenary sessions then focused on three major scientific themes: 1) in vitro replacement methods for potency testing, 2) refinement and reduction of animal use for potency testing, and 3) reduction, refinement, and replacement alternatives for safety testing. Each day, the plenary session was followed by breakout group discussions on the respective theme for veterinary and human vaccines. Recommendations arising from the breakout groups were then presented in the plenary session for further discussion and consensus.

3. The importance of vaccines to human and animal health

In the opening plenary session, Drs. Anne Schuchat and James Roth described how vaccines have proven to be an extremely cost effective approach for preventing infectious diseases in people and animals [8, 10]. For example, smallpox has been globally eradicated, and many other diseases such as polio, measles and rubella are now only rarely found in North America. Pediatric vaccinations (e.g., diphtheria, tetanus, pertussis, measles, mumps, rubella, polio) save an estimated 43 billion dollars in medical and societal costs annually [8, 9].

Veterinary vaccines prevent a wide range of infectious diseases in companion animals, livestock, wildlife, fish, zoo animals, and many other animal populations. Veterinary vaccines also contribute to human health by controlling zoonotic diseases that can be transmitted from animals to humans, such as rabies. They serve a vital role in controlling and preventing foreign animal diseases in many countries, such as foot and mouth disease (FMD) and emerging diseases such as new influenza strains. Rinderpest disease, a devastating livestock disease that has persisted for thousands of years, has now been eradicated worldwide due to the availability and implementation of effective veterinary vaccines [10, 11]. To help reduce foodborne diseases in people caused by Salmonella, layer hens can be vaccinated to prevent Salmonella-infected eggs.

4. Vaccine potency and safety testing regulatory requirements

Representatives from the U.S. Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA) joined scientists from Europe, Canada, Japan, and the World Health Organization (WHO) to discuss their regulatory requirements and guidelines for ensuring the safety, purity, potency, and efficacy for human and veterinary vaccines [12, 13, 14, 15, 16, 17]. Prior to their release, all vaccine lots are tested to verify that they are safe, pure, sufficiently potent, and effective. Such testing ensures that post-approval production of each lot of vaccine maintains the antigenic characteristics that result in their effectiveness.

The speakers highlighted that while testing of some vaccines can be accomplished with few or no animals, testing of many vaccines still requires the use of animals. For example, immunization-serology or immunization-challenge procedures in laboratory or target animals are commonly used to demonstrate potency. Animals are also used for post-licensing safety testing to prevent the release of lots that might cause serious adverse health effects [12, 13]. Due to the significant number of animals used globally for vaccine lot release testing and the potential for unrelieved pain and distress, many national and international vaccine manufacturers and regulatory agencies as well as organizations such as the World Health Organization have targeted efforts to minimize the use of animals for such testing requirements. Alternative approaches to vaccine potency and safety testing methods include: reduction in the total number of animals required for a procedure, refinement by using earlier humane endpoints or using serology to replace challenge testing, and replacement of animals with in vitro antigen quantification methods.
Speakers in subsequent plenary sessions provided examples of progress that has been made in reducing, refining, and replacing animal use for vaccine potency and safety testing. Reduction in animal numbers has been achieved by optimizing testing procedures to reduce or avoid sources of experimental variables, thereby reducing the need for multiple vaccine dilution testing in some testing situations [18, 19, 20]. With regard to refinement, humane endpoints have been defined and implemented that allow an in vivo test to be ended sooner, thus reducing the severity and/or duration of pain and distress without compromising the initial study objective [19, 21, 22, 23]. Refinement has also been accomplished by using antibody quantification, or serological tests, to replace challenge testing with virulent organisms. Serologic tests offer a number of advantages, including: reducing the distress and number of animals used; improving the safety of the laboratory worker; reducing the time to perform the test; and using a quantitative instead of qualitative end point [20, 24, 25, 26, 27]. Finally, the most promising replacement alternatives are antigen quantification assays. However, these methods require knowledge of the protective antigen(s) and in many cases, removal of the adjuvant from the product prior to antigen quantification [19, 28, 29].

5. Priority vaccines for future research, development and validation activities

During the breakout sessions, workshop participants developed criteria that should be used to prioritize human and veterinary vaccines for further refinement, reduction, and replacement (3Rs) of potency testing procedures (Table 1). Prioritization criteria included vaccines with potency testing procedures that involve severe animal pain and distress, that use the largest number of animals, and those for which alternative methods have been developed but are not yet validated or implemented (Table 1). Additional criteria include vaccines involving pathogens that can be easily spread to wildlife populations or that are considered foreign animal/transboundary diseases, and those that require the use of live infectious agents that are hazardous to laboratory workers.

The criteria were then used to identify and prioritize human and veterinary vaccines for further 3Rs research, development, validation, and implementation activities (Table 2). The highest priority human vaccines include those used to prevent diphtheria, tetanus, pertussis, rabies, anthrax, and polio, and combination vaccines primarily used for pediatric immunizations. The highest priority veterinary vaccines include those used to prevent diseases caused by rabies, Clostridium species, and Leptospira interrogans serovars, as well as foreign animal/transboundary diseases, and poultry and fish diseases (Table 2).

Workshop participants also provided recommendations and initiatives for achieving broader acceptance and use of 3Rs methods for both human and veterinary vaccine potency and safety testing. There was consensus agreement among workshop participants that there needs to be broader access to information; increased interaction/communication between worldwide regulatory agencies, research institutions, and vaccine manufacturers; and harmonization of national requirements, methods and specifications that would facilitate the broader acceptance and use of alternative methods for vaccine potency testing of human and veterinary vaccines.

6. Vaccine potency testing: replacing animals with non-animal methods

Speakers in the second plenary session highlighted inactivated vaccines where non-animal methods using in vitro antigen quantification procedures have replaced the use of animals for vaccine potency testing. [2, 3, 19, 28, 29, 30, 31]. For human vaccines, these are typically antigen quantification methods based on the binding of key protective antigens in the vaccine batch to specific reference antibodies in an in vitro immunoassay. Examples include Hepatitis A and B vaccines, inactivated polio vaccine, and human papillomavirus vaccine [24, 30]. For well characterized polysaccharide vaccines, including Streptococcus pneumoniae, Haemophilus influenzae, Salmonella typhi and Neisseria meningitidis, specific in vitro physiochemical characteristics have been used to correlate to vaccine potency without the need for animal testing [31].

For veterinary vaccines, several types of in vitro methods have been developed for potency testing of live and inactivated vaccines [29]. Antigen quantification methods are used for some inactivated bacterial vaccines or bacterins such as E. coli bacterins [32, 33, 34, 35], and for some Leptospira interrogans serovars, including pomona [36], canicola [37, 38], grippotyphosa [39], and hardjo [40]. Antigen quantification methods have also been developed for inactivated viral vaccines such as feline Leukemia Virus Vaccine [41] and Newcastle Disease vaccine [28, 42].
Other types of non-animal alternatives are available for live or modified live veterinary vaccines. For example, for modified live bacterial vaccines, live organisms are quantified by colony forming unit (CFU) enumeration methods (e.g., Brucella abortus [43]. For some live viral vaccines, virus is quantified by observing cytopathic effects in cell cultures (e.g., porcine transmissible gastroenteritis vaccine [44] and Porcine Rotavirus [45]). Modified live viral vaccines such as feline panleukopenia [46] and canine Parvovirus [47] utilize direct or indirect fluorescent antibody staining of virus inoculated cell cultures to quantify virus titers.

Table 1. Criteria to prioritize human and veterinary vaccines for future 3Rs methods and achievement of broader acceptance and use

<table>
<thead>
<tr>
<th>Criteria for Prioritization</th>
<th>1. Vaccines that require an in vivo challenge test and/or involve severe pain and distress</th>
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<tbody>
<tr>
<td></td>
<td>2. Vaccines that require the largest number of animals based on both the total number of animals required per test and the number of batch release tests conducted</td>
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<tr>
<td></td>
<td>3. Vaccines for which alternative methods are already developed, but not validated or implemented</td>
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<td></td>
<td>4. Vaccines for which the functional protective antigen has been identified and characterized</td>
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<tr>
<td></td>
<td>5. Vaccines with in vivo tests that are highly variable and often require repeat testing</td>
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<tr>
<td></td>
<td>6. Vaccines involving zoonotic or other pathogens that are dangerous to humans</td>
</tr>
<tr>
<td></td>
<td>7. Vaccines involving pathogens that can be easily spread to wildlife populations or that are considered foreign animal/transboundary diseases</td>
</tr>
</tbody>
</table>

Table 2. Priority vaccines for future 3Rs methods and achievement of broader acceptance and use

<table>
<thead>
<tr>
<th>Human Vaccines</th>
<th>Veterinary Vaccines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diphtheria and tetanus toxoids</td>
<td>Rabies</td>
</tr>
<tr>
<td>Pertussis vaccines (whole cell and acellular)</td>
<td>Leptospira interrogans serovars</td>
</tr>
<tr>
<td>Rabies vaccines</td>
<td>Clostridium species</td>
</tr>
<tr>
<td>Anthrax vaccines</td>
<td>Erysipelas</td>
</tr>
<tr>
<td>Combination vaccines such as diphtheria/tetanus/pertussis (DTP)-based pentavalent vaccines</td>
<td>Foreign animal/transboundary disease vaccines</td>
</tr>
<tr>
<td>Inactivated polio vaccines</td>
<td>Poultry vaccines</td>
</tr>
<tr>
<td></td>
<td>Fish vaccines</td>
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</table>

Workshop participants discussed and developed recommendations for further advancing non-animal replacement methods for vaccine potency testing for human [2] and veterinary [3] vaccines. To facilitate the successful development of in vitro replacement alternatives for vaccines, further research is required to understand the immunological mechanisms of vaccine protection as well as to identify the relevant protective antigen(s) in the vaccines. In vitro quantification of antigens must be correlated to biological activity in terms of immunologic protection for successful potency test replacement [19, 30]. For many veterinary vaccines, the protective antigen is unknown or is a complex combination of antigens [29]. To advance non-animal potency testing methods, additional research and development efforts are needed to identify, purify, and characterize the specific vaccine protective antigens in vaccines (Table 3).

Many vaccines, especially veterinary vaccines, often contain adjuvants, which are used to enhance the immune response to the vaccine. However, some commonly used adjuvants, such as mineral oil and aluminum salts, may interfere with in vitro quantification methods by modifying the antigen of interest. An in vivo potency test assesses the protective immune response to the complete vaccine, which includes antigenic material, adjuvants and excipients. A replacement in vitro method would therefore need to accurately characterize when an adjuvanted vaccine was able to similarly provide a protective response. Accordingly, there is an urgent need for further research and development on separation methodology to extract the protective antigen from adjuvants to avoid adjuvant interference in subsequent antigen quantification assays [29] (Table 3).
Table 3. Priority research needs to develop in vitro replacement alternatives for human and veterinary vaccine potency testing

<table>
<thead>
<tr>
<th>Replacement Methods</th>
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<tbody>
<tr>
<td></td>
<td>Improve understanding of the immunological mechanisms of vaccine protection</td>
</tr>
<tr>
<td></td>
<td>Identify clinically relevant protective antigens</td>
</tr>
<tr>
<td></td>
<td>Identify, purify, and characterize vaccine protective antigens</td>
</tr>
<tr>
<td></td>
<td>Develop separation methodology to extract the protective antigen from adjuvants to avoid adjuvant interference in subsequent antigen quantification assays</td>
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</table>

7. Refinement alternatives for vaccine potency testing

The third plenary session included presentations on refinement strategies that could be used to lessen or avoid pain and distress in animals used for human and veterinary potency testing procedures, and reduction strategies to reduce the numbers of animals needed for each potency test [18, 25, 26, 27, 48, 49, 50, 51]. Refinement focused on two approaches. The first is the use of earlier more humane endpoints to lessen pain and distress when animals are used for challenge testing or toxin neutralization testing. The second is the use of serologic assays to avoid the need for challenge testing, and the use of serologic assays together with in vitro toxin neutralization assays. A brief summary of the workshop recommendations for further refinement and reduction of animal use for vaccine potency testing is provided below, while more detailed discussions are provided in separate workshop reports for human [4] and veterinary [7] vaccines.

7.1. Using humane endpoints to refine animal use

The potency of many vaccines is assessed using an immunization-challenge procedure in laboratory animals or the target species, which can involve significant pain and distress. In this procedure, serial dilutions of the vaccine are administered to several groups of animals. A group of control animals is included that do not receive vaccine. All animals are then challenged with a dose of live pathogens sufficient to cause clinical disease in unprotected animals in order to determine the quantity of vaccine that provides protection. Inadequately protected and unprotected control animals typically develop clinical disease or die.

When vaccination-challenge tests are still required, humane endpoints can be used as the basis for ending studies earlier than death or severe disease in order to decrease the severity and duration of pain and distress experienced by animals. Earlier humane endpoints are clinical signs or other objective measurements such as body temperature changes that are indicative of the final study endpoint, but that allow for earlier termination of studies while still meeting experimental objectives [4,5]. Using humane endpoints to reduce animal pain and distress is reflected in regulatory documents and the legislation of relevant agencies in numerous regions and countries worldwide [21, 23, 48, 52].

Workshop participants agreed that humane endpoints should be identified and implemented for all challenge testing. The identification of humane endpoints involves the initial routine collection of all clinical signs and other candidate measurements during challenge-testing procedures. This data is then analyzed to identify the parameters and their severity that are predictive of the current experimental endpoint, which for potency challenge tests is usually moribund condition or death. The workshop recognized that comprehensive training in the recognition of clinical signs, which may occur during a challenge test, is essential. Additional refinements can be achieved through the use of anesthetics for intracranial injections or other potentially painful procedures, and ensuring adequate training of laboratory personnel involved in injection and challenge procedures [53, 54, 55, 56] (Table 4).

7.2. Using serological methods to refine animal use

The other major strategy for avoiding pain and distress is to use vaccination-serology methods as an alternative to vaccination-challenge methods for potency determination [4,5]. Serological methods quantify the specific antibody levels in the blood of immunized animals. These results are then compared to the antibody titer previously shown to correspond to protection against challenge by the relevant pathogen. Examples include two serological methods
developed for rabies vaccines, that quantify rabies virus neutralizing antibodies in serum from immunized animals [19]. The methods include the rapid fluorescent focus inhibition test (RFFIT) [57, 58] and the fluorescent antibody virus neutralization test (FAVN) [59].

Human tetanus and diphtheria vaccines have traditionally used a guinea pig lethal/paralytic challenge test. However, serological assays are now in use that have been approved by some regulatory authorities. Antibody responses are measured using the toxin-binding inhibition (ToBI) or ELISA methods for tetanus [20, 25, 26, 60] and either an ELISA or a Vero cell toxin neutralization test for diphtheria [26, 61].

Serological alternatives for potency testing have been developed and accepted for a number of veterinary vaccines. Examples include two serology methods for tetanus, an ELISA assay [63] and a toxin-binding inhibition test [64]. A serology and ELISA assay for inactivated *Erysipelothrix rhusiopathiae* vaccine is also approved [65, 66]. Serological techniques can also be used for the potency release of vaccine serials for several *Leptospira interrogans* serovar vaccines, including canine leptospirosis [67] and bovine leptospirosis vaccine [40].

Another successful approach used for some veterinary vaccines is the use of in vitro toxin neutralization tests (TNT) to replace in vivo TNTs, which typically are conducted using mice. In these tests, the protective antibody generated in a group of animals is combined in vitro with known amounts of toxin produced by the causative agent (e.g., *Clostridium* spp. vaccines). This mixture is then administered to laboratory animals to assess whether the protective antibody fully neutralized the toxin. The presence of residual toxin is indicated by abnormal appearance or death of the animals.

Research and development efforts have led to in vitro methods, such as an indirect ELISA, that can be used to quantify the protective antibody without the need for in vivo TNT testing. For example, in vitro methods are available that can be used in place of in vivo TNT for *Clostridium perfringens* [68] *Clostridium septicum* [69], and *Clostridium novyi* [70, 71].

Workshop participants identified and recommended priority research and other activities necessary to further develop reduction and refinement alternatives for human and veterinary vaccine potency testing (Table 4 and Table 5). In addition, they emphasized that to facilitate the identification and use of humane endpoints, clear guidance on the recognition of clinical signs, especially those indicative of moribund condition, need to be included in comprehensive training of laboratory personnel. The validation, acceptance, and implementation of serological tests for both human and veterinary rabies vaccines were highlighted as an important near term priority. Accordingly, workshop participants recommended a future workshop to focus specifically on the assessment of rabies vaccine serological methods as substitutes for the current in vivo challenge methods. Other high priorities were the continued development and validation of serological methods for several *Leptospira interrogans* serovar vaccines, and the development and validation of ELISA or cell based assays for the in vivo Toxin Neutralization Test (TNT) for several *Clostridium* spp. vaccines (Table 4).

### 8. Reduction alternatives for vaccine potency testing

Where animals must still be used for potency testing, workshop participants discussed how the number of animals has been reduced, and recommended ways to potentially further reduce the number of animals used in each potency test. One approach is to limit the number of dose groups when it can be scientifically justified. For example, single dilution assays have been proposed instead of traditional multiple dilution challenge procedures for human and veterinary rabbit vaccines and the modified PPD tuberculin test in guinea pigs [18, 19, 27]. Another example is the use of a common serological approach for vaccines, which are often combined, such as diphtheria and tetanus vaccines, thereby avoiding the need for separate potency assays for each component of the vaccine [20, 25].

### 9. Alternatives for vaccine safety testing

In the fourth and last plenary session, speakers addressed the current regulatory requirements and rationale for post-licensing safety testing of human and veterinary vaccines [12, 72], and discussed strategies for reducing, refining, and replacing animal use for vaccine safety testing [49, 73]. Vaccine safety implies the absence of residual virulence for live attenuated vaccines, freedom from extraneous agents, and the absence of toxicity [12, 74]. Vaccine safety is maintained by extensive testing, the use of manufacturing and monitoring processes to ensure consistent quality, and post-marketing surveillance. Safety testing typically includes a general safety test to evaluate the
potential for any systemic toxicity that might be associated with the vaccine or any of its components, and to ensure absence from extraneous toxic contaminants that may be introduced in the final containers through undetected failures in the manufacturing process. Depending on the vaccine, there may be requirements for additional specific safety tests, such as testing to detect reversion of inactivated toxins to their virulent form, neurovirulence tests for live attenuated vaccines (e.g. oral polio vaccine), tests for pyrogenic (fever-producing) substances, and extraneous agent tests to detect viral or other microbial contamination [7, 8, 12].

Table 4. Recommendations for expanding the use of humane endpoints and serological methods to lessen or avoid pain and distress in human and veterinary vaccine potency testing

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>• Earlier humane endpoints should be identified and implemented for all vaccines requiring challenge testing.</td>
<td>• Conduct research needed to identify antibodies and immune processes necessary for protective immunity.</td>
</tr>
<tr>
<td>• All clinical signs that occur during a challenge test should be routinely collected and evaluated to identify earlier humane endpoints.</td>
<td>• Conduct research to develop methods to assess functionality of antibodies or other immune responses.</td>
</tr>
<tr>
<td>• Clinical observations and other relevant measurements should be collected and analyzed during premarketing efficacy tests to identify humane endpoints that can be implemented for lot release potency testing.</td>
<td>• Develop and validate assays and reagents to quantify antibodies in the serum of vaccinated animals.</td>
</tr>
<tr>
<td>• Comprehensive training on clinical signs likely to occur during a challenge test that should be collected or used for potential humane endpoints should be provided to all personnel conducted such testing.</td>
<td>• Implement ELISA and toxin binding inhibition (TOBI) assays for measuring antibodies to tetanus toxoid.</td>
</tr>
<tr>
<td>• Threshold percentages should be established for control animals in challenge tests that have reached the specified endpoint for euthanasia that could then be used for euthanasia of remaining controls.</td>
<td>• Implement the Vero cell assay and ELISA for measuring protective antibodies to diphtheria toxoid.</td>
</tr>
<tr>
<td>• Earlier human endpoints should be more readily identified and implemented for vaccines for which animals take a longer period of time to develop disease (e.g., vaccines for <em>Leptospira interrogans</em> serovars).</td>
<td>• Convert <em>in vivo</em> toxin neutralization tests to ELISA or other cell-based methods for appropriate <em>Clostridium spp.</em> Vaccines.</td>
</tr>
<tr>
<td>• Animals used in challenge testing should be monitored at least twice daily for moribund condition or evidence of an established humane endpoint, or more often as necessary to minimize or preferably avoid spontaneous deaths.</td>
<td>• Convene focused working group and workshop involving both human and veterinary rabies researchers.</td>
</tr>
<tr>
<td>• Innovative methods to observe animals more readily for earlier humane endpoints should be developed, including techniques that will further avoid stress from human contacts.</td>
<td>• Conduct further research and validation of rabies serological methods to gain acceptance for human vaccine lot release.</td>
</tr>
<tr>
<td>• Detailed institutional protocols and guidance documents for identification and use of humane endpoints should be developed.</td>
<td>• Conduct research and validation of the immunogenicity test to measure antibody response to anthrax human vaccine.</td>
</tr>
</tbody>
</table>
Table 5. Recommendations for reducing the use of animals in human and veterinary vaccine potency testing

<table>
<thead>
<tr>
<th>Reduction Strategies</th>
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<tbody>
<tr>
<td>• Systematically identify and reduce or eliminate the sources of excessive variation in order to reduce the number of animals per dose group</td>
</tr>
<tr>
<td>• Systematically review and determine the basis for inconclusive test results requiring repeat testing, with the goal of reducing the frequency of repeated tests</td>
</tr>
<tr>
<td>• Retrospectively review archival data to determine if the minimum number of animals (including control animals) can be reduced while maintaining the necessary statistical power for current tests</td>
</tr>
<tr>
<td>• Investigate how to further reduce the number of animals required for diphtheria and tetanus potency testing</td>
</tr>
<tr>
<td>• Incorporate flexibility into the regulatory process so that the reduction of animals can be applied on a case-by-case basis, particularly for minor-use situations</td>
</tr>
<tr>
<td>• Use a product-specific reference to reduce variability and improve precision</td>
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</tbody>
</table>

While a general safety test (GST) for human and veterinary vaccines is required by some authorities, exemptions to the GST may be granted for certain licensed products if specific manufacturing processes and controls are instituted that consistently demonstrate safety, purity and potency. Similarly, the abnormal toxicity safety test (ATT) for human vaccines and the target animal safety test for veterinary vaccines may be waived in instances where there is demonstration of consistency in production and assurance that vaccines were produced in accordance with the principles of GMP and quality assurance [71, 74, 75]. While process changes or other modifications that could alter the product may require that these tests again be performed, such exemptions can dramatically reduce the number of animals required for some vaccine safety testing.

For some vaccines containing an inactivated toxin (e.g., Diphtheria, Tetanus, acellular Pertussis (aP) and whole-cell Pertussis (wP)) or inactivated virus (e.g., Rabies), testing is required to ensure complete inactivation prior to lot release [49]. An in vitro Vero cell assay has been developed for detection of diphtheria toxin and is currently in use in Europe and approved by the WHO. However, the test cannot be used with vaccines using absorbed diphtheria toxoids and will require further research to enable broader international use [76]. For the detection of active pertussis toxin (PT) in either aP or wP vaccines, alternative tests are under development to replace the Mouse Weight Gain test for wP or the histamine sensitization assay for aP. However, research and validation efforts are still required to adequately detect PT in vaccines containing adjuvants, such as aluminum salts, or preservatives [49].

Molecular methods (e.g., polymerase chain reaction [PCR]) have been developed that can replace animals for extraneous agent testing for both live and inactivated poultry vaccines [77, 78, 79]. Results indicate that the sensitivity of the PCR procedure is at least equal to or in most cases higher than that of conventional in vivo testing. PCR tests are now available for 15 different extraneous agents tested in avian vaccines including avian adenoviruses, avian infectious bronchitis virus, chicken anemia virus, infectious bursal disease virus, and Newcastle Disease virus [80].

Workshop participants identified vaccines that should have priority for efforts to further reduce, refine, and replace the use of animals for post-licensing safety testing (Table 2), and identified knowledge gaps and priority research necessary to advance alternatives for post-licensing safety tests for human [6] and veterinary vaccines [7] (Table 6). Among the highest priority activities is further assessment of the need for the general safety test, and to explore international harmonization of the specific circumstances for waiving the test. Additional priorities included research on applying cell culture and PCR techniques to further replace in vivo chicken tests for extraneous agents, and determining if the in vivo veterinary rabies inactivation test could be replaced with currently available cell culture techniques for testing virus inactivation for human rabies virus (Table 6).

Workshop participants also recommended that in-process safety testing should be used to verify detoxification of selected vaccines, and that cell based assays should be developed that can measure residual toxicity. Participants also agreed that achieving broader acceptance of alternative safety tests required both targeted specific research efforts as well as broad efforts to improve communication, collaboration and harmonization of developed procedures.

To advance international harmonization, further discussion was recommended to obtain agreement on how consistency in manufacturing processes can be adequately demonstrated and to develop international criteria for
consistency measures. Continued interaction of the global vaccine community within both human and veterinary sectors was recommended to expedite the unified goal of identifying alternatives to animal testing for vaccine safety testing.

Table 6. Priority research needs for the development of 3Rs alternative post-licensing safety tests for human and veterinary vaccines

<table>
<thead>
<tr>
<th>Priority Research Needs</th>
<th>Human vaccines:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>• Refinement of the acellular pertussis lethal endpoint histamine sensitization assay (HSA) to a dermal temperature endpoint</td>
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<tr>
<td></td>
<td>• Selection and validation of combined <em>in vitro</em> assays as replacement alternatives to HSA (e.g., chromatographic separation and measurement of an ADP-ribosylated fluorescent substrate)</td>
</tr>
<tr>
<td></td>
<td>• Development of <em>in vitro</em> assays to detect residual pertussis toxin</td>
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<tr>
<td></td>
<td>• Research to expand the use of the transgenic mouse model for neurovirulence testing of oral live polio vaccines</td>
</tr>
<tr>
<td></td>
<td>• Expanded efforts for the sequence-based approach to oral polio vaccine neurovirulence safety testing</td>
</tr>
<tr>
<td></td>
<td>• Development of alternatives to the monkey neurovirulence test for preclinical safety and lot release neurovirulence testing of mumps (and possibly measles) vaccines</td>
</tr>
<tr>
<td>Veterinary vaccines:</td>
<td>• Continued investigation of cell culture and PCR techniques to replace <em>in vivo</em> chicken tests for extraneous agents</td>
</tr>
<tr>
<td></td>
<td>• Determining if the <em>in vivo</em> veterinary rabies inactivation test could be replaced internationally with cell culture techniques for the testing of virus inactivation for human rabies virus vaccines</td>
</tr>
<tr>
<td>Human and Veterinary Vaccines:</td>
<td>• Expanded use of the Vero cell assay to monitor diphtheria toxin inactivation</td>
</tr>
<tr>
<td></td>
<td>• Development of a fully functional <em>in vitro</em> assay for tetanus toxin</td>
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<tr>
<td></td>
<td>• Investigation into how to develop, validate, and implement safety testing using a series of various tests in a consistency approach</td>
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<tr>
<td></td>
<td>• Assessment of the need for the general safety test and determining internationally harmonized criteria for when the test may be waived</td>
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10. International harmonization, acceptance, and implementation of alternative methods for vaccine potency and safety testing

Workshop participants recognized and agreed that there is a strong commitment on the part of national and international regulatory agencies and organizations, vaccine manufacturers, and animal welfare agencies to reduce, refine, and replace animals in vaccine potency and safety testing. Workshop participants recommended several ways to accelerate and achieve international harmonization and acceptance of alternative methods for human and veterinary vaccine potency and safety testing (Table 7).

Participants acknowledged the important role of international organizations in achieving global harmonization and implementation of alternative methods. For human vaccines, the World Health Organization serves a critical role in not only developing consensus guidelines, but also in providing critical reagents for potency and safety testing. For veterinary vaccines, the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH) is a trilateral program of collaboration among the regulatory authorities and animal health industries of the European Union, Japan, and the United States. VICH works in collaboration with
the World Animal Health Organization to establish and implement specific guidelines with input from the international scientific community, which results in broad-based review and acceptance.

Table 7. Recommendations for achieving international harmonization and acceptance of alternative methods for vaccine potency and safety testing

<table>
<thead>
<tr>
<th>Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>National requirements, methods and specifications should be harmonized internationally to facilitate broader acceptance and use of alternative methods for vaccine potency testing of human and veterinary vaccines</td>
</tr>
<tr>
<td>There should be international harmonization of both regulatory requirements and test method acceptance criteria along with increased transparent communication between regulators, manufacturers, and the scientific community.</td>
</tr>
<tr>
<td>There should be broader, open access to information on available alternative methods that are currently in use, and access to non-proprietary reagents, antibodies, and reference standards.</td>
</tr>
<tr>
<td>Enhanced international harmonization and cooperation and closer collaborations between human and veterinary researchers is recommended to facilitate progress in both the human and veterinary vaccine sectors</td>
</tr>
<tr>
<td>Further international discussions and harmonization are recommended for defining and measuring consistency parameters in vaccine manufacturing processes</td>
</tr>
<tr>
<td>A future international workshop is recommended to review the validation status and implementation approaches for serological methods for rabies vaccine potency testing</td>
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</table>

11. Conclusions

This was the first international workshop held in the United States that focused on the replacement, refinement, and reduction of animal use for vaccine potency and safety testing for both human and veterinary vaccines. The workshop provided a unique opportunity for interested stakeholders from the human and veterinary vaccine fields to interact and gain important insights on the current similarities and differences in vaccine potency and safety testing procedures across each region.

There was an obvious benefit and synergy created by the participation and interactions between experts from industry, academia, and government. As such, there was broad recognition among participants that the workshop provided an important opportunity for information exchange not only between global regions but also between regulatory authorities. Continuing the communication, collaboration, and cooperation established at this workshop will undoubtedly help advance the development and implementation of alternative methods for vaccine potency and safety testing.

The workshop reviewed the current state of the science for alternative methods that can reduce, refine, and replace animal use for human and veterinary vaccine potency and safety testing. Numerous methods were identified that are available and are accepted by regulatory authorities. Use of these methods by vaccine manufacturers will have an immediate impact on reducing animal use and providing for more humane use of animals.

For many methods, additional research, development, and validation efforts will be necessary to standardize and determine the scientific validity of the methods for regulatory purposes. As new science and technologies become available, there will undoubtedly be enhanced opportunities for future progress in terms of not only more efficient methods, but also more accurate methods. However, the rate of future progress will depend in large part on the availability and commitment of resources to conduct the studies necessary to advance alternative methods.

The reports and recommendations from this workshop should prove useful to stakeholders interested in further reducing, refining, and replacing animal use for vaccine testing. Implementation of the workshop recommendations is expected to advance alternative methods for vaccine potency testing that will benefit animal welfare while ensuring safe and effective vaccines to protect people and animals.
Acknowledgements

The authors extend their sincere appreciation to all participants in the international workshop for their contributions leading to the workshop conclusions and recommendations. The members of the ICCVAM Interagency Biologics Working Group and NICEATM staff are acknowledged for their contributions to the planning of the workshop, and the many invited experts are acknowledged for their contributions to breakout group discussions and workshop proceedings. Finally, the authors thank David Allen, Vivian Doelling, Nelson W. Johnson, and Brett Jones for their assistance in the preparation of this manuscript.

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